ISOLATION OF PEPTIDES WITH IMMUNOREACTIVITY FROM OVALBUMIN BY TRYPSIN DIGESTION

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1. Introduction

It was reported that on denaturation ovalbumin loses to a considerable extent the ability to react with antibodies against native ovalbumin [1,2]. Although many investigators tried to isolate peptides with antigenicity from ovalbumin by enzymic and chemical degradations, there was not any evidence of production of fragments with immunoreactivity [3,4]. These facts imply that the conformation of ovalbumin is responsible to its antigenicity. It is known that native ovalbumin is not digested with trypsin or chymotrypsin at all, owing to the rigid conformation of ovalbumin in native state [4]. In [5], the significance of tyrosine residues in ovalbumin was pointed out as a specific amino acid responsible for antigenic determinants by chemical modification study.

We demonstrate here a novel approach for rendering ovalbumin susceptible to hydrolysis with trypsin and were able to obtain two peptides with immunoreactivity. Peptides B and A thus obtained were single polypeptides stretching from N-terminal $AcGly_1$ to Lys_{189} with M_r 23 000 and from Leu_{105} to C-terminal Pro_{385} containing polysaccharide with M_r 34 000. Both peptides had an ability to bind with anti-ovalbumin serum.

2. Materials and methods

Ovalbumin (crystallized, lyophilized, salt and ovomucoid-free) and trypsin inhibitor from soy bean (type II-S) were obtained from Sigma Chemical Co. (St Louis MO). Crystalline porcine trypsin (dialyzed and lyophilized) was obtained from Novo (Copenhagen). Ovalbumin (10 ml, 100 mg) in 0.1 M borate buffer (pH 8.0), 10 ml 1% sodium dodecyl sulfate (SDS) and 20 ml 70% glycerol were mixed well. To the mixture (40 ml) 10 ml buffer A was added which contained 0.1 M borate buffer (pH 8.0) including 10 mM EDTA and 1 mM mercaptoethanol. Then 10 ml trypsin (5 mg) in buffer A was added to 50 ml above ovalbumin solution and kept standing for 50 h at 20°C. To obtain the peptides with immunoreactivity, digestion of ovalbumin with trypsin should be carried out with 0.17% SDS and 23% glycerol. Then, the sample solution was dialyzed against buffer A after the addition of immobilized trypsin inhibitor, and the immobilized trypsin inhibitor bound to trypsin was removed by filtration. Trypsin inhibitor was immobilized to Sepharose 4B as in [6]. The sample protein solution was concentrated by ultrafiltration with a membrane (G-05T) and subjected to gel chromatography with Sephadex G-100 column (2.5 × 90 cm) equilibrated with buffer A containing 0.1% SDS. Peptides thus obtained were dialyzed against phosphate-buffered saline (PBS) to remove SDS completely and were subjected to the following experiments.

Electrophoresis of ovalbumin and its peptides was carried out in 10% (w/v) polyacrylamide gel containing 0.1% SDS as in [7]. The gels were stained in 1% amide black 10B in 7% acetic acid and destained with 7% acetic acid.

White rabbits were immunized with native ovalbumin (1 mg) in complete Freund's adjuvant by subcutaneous injection once a week for 4 weeks. In the second week after the last injection, the antisera were obtained and stored at −20°C. A double diffusion study was performed by the standard method [8].

N-Terminal amino acid residues in peptides were identified with phenylisocryanate [9], using two-dimensional, thin-layer chromatography with polyacrylamide [10]. Amino acid composition was determined with a JEOL JLC-6AH amino acid analyzer

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after hydrolysis of a protein with 6 N HCl at 110°C for 22 h. Content of carbohydrate was determined by means of phenol–sulfuric acid reaction [11].

3. Results and discussion

Fig.1 shows the Sephadex G-100 chromatogram of ovalbumin digested with trypsin in the presence of 0.17% SDS and 23% glycerol. Three components were obtained which are called fractions A, B and C in the order of elution. Fraction C contained small peptides with $M_r < 10\,000$. Further purification of proteins present in fractions A and B were carried out by gel chromatography under the same experimental conditions as above, after separately collecting the protein solutions obtained in tubes 60–77 and 78–85, respectively. This procedure was repeated 3 times to obtain a protein in a homogeneous state. Elution patterns of fractions A and B obtained by the third chromatography with Sephadex G-100 have a single sharp band, indicating that two different proteins named peptides A and B were isolated in a pure state from fractions A and B, respectively. Disc electrophoretic pattern of peptides A and B show also a single sharp band and their M_r-values were estimated to be 34 000 (peptide A) and 23 000 (peptide B) (fig.2).

The primary structure of ovalbumin determined in [12] consists of 385 amino acid residues from N-terminal AcGly to C-terminal Pro. To assign the position of peptides A and B on the primary structure, amino acid composition, sugar content and N-terminal amino acid residue were determined. Table 1 shows the amino acid compositions of peptides A and B, which are in fairly close agreement with the sequence of amino acid residues in ovalbumin from Leu_{105} to C-terminal Pro_{385} and from N-terminal $AcGly_1$ to Lys_{189}, respectively. N-Terminal amino acid residue of peptide A was determined to be Leu but that of peptide B could not be detected, suggesting the masking of an N-terminal amino group. The contents of carbohydrate of peptides A and B were determined to be 3.5% and 0.3% in weight, respectively. Ovalbumin is a glycoprotein which contains 3.2% carbohydrate binding to the position of Asp_{292}. Peptides B and A can be thus unequivocally assigned to positions 1–189 and 105–385, respectively.

Immunochemical activity between anti-ovalbumin serum and peptide A or B was studied by immunodiffusion. It was confirmed that urea-denatured ovalbu-

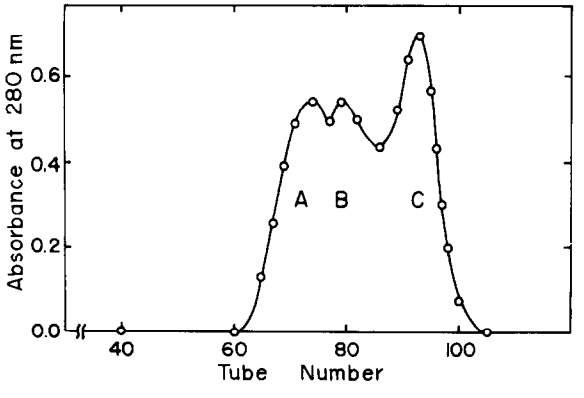

Fig.1. Gel chromatogram of ovalbumin digested with trypsin in the presence of SDS and glycerol, using Sephadex G-100 (2.5 × 90 cm) equilibrated with buffer A containing 0.1% SDS. Elution was performed with buffer A containing 0.1% SDS.

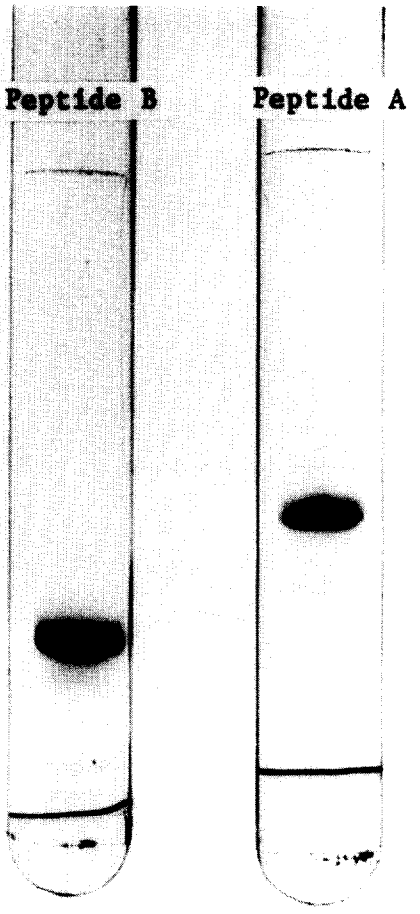

Fig.2. Disc electrophoretic pattern of peptides A and B.

Table 1
Amino acid composition of peptides A and B together with ovalbumin (residues/mol)

Amino acid	Peptide A		Peptide B		Ovalbumin	
	Found	Calc. (105–385)	Found	Calc. (1–189)	Found	Calc. (1–385)
Lys	14.5	14	10.3	10	21.5	20
His	4.0	4	3.0	3	7.0	7
Arg	11.7	11	8.1	8	15.5	15
Asp	22.8	19	18.9	19	34.3	31
Thr	11.2	11	6.7	7	15.6	15
Ser	24.8	27	16.1	16	35.0	38
Glu	40.7	41	23.2	23	50.7	48
Pro	11.2	11	6.8	7	15.0	14
Gly	13.0	13	10.4	10	20.3	19
Ala	25.6	26	16.7	15	35.0	35
Val	19.8	23	12.5	14	26.9	31
Met	13.0	13	5.8	4	15.7	16
Ile	16.2	17	12.3	14	23.0	25
Leu	25.4	25	15.6	16	32.4	32
Tyr	7.7	7	6.3	7	9.4	10
Phe	14.3	13	10.4	10	20.0	20
Trp[a]	2.5	3	1.8	2	3.0	3

[a] Tryptophan content was determined spectrophotometrically as in [13]

min (8 M, 30 h) does not show a precipitin arc against anti-ovalbumin serum by immunodiffusion analysis. Immunodiffusion patterns of peptides A and B against anti-ovalbumin serum is shown in fig.3. A strong precipitin line was observed between native ovalbumin and anti-ovalbumin serum. The line was partially fused with another precipitin line between peptide A (or peptide B) and anti-ovalbumin serum and strong spurs appeared as seen in fig.3. Two precipitin lines obtained between anti-ovalbumin serum and peptides A and B were almost completely fused, without any spurs. Denaturation of each peptide caused the disappearance of precipitin line. These results indicate that the conformations of peptides A and B responsible for antigenic determinants are still retained even when ovalbumin is digested with trypsin and that a part of the antigenic determinants in ovalbumin exists in the common sequence of amino acid residues in peptides A and B stretching from Leu_{105}–Lys_{189}. A peptide from Leu_{105}–Lys_{189} contains 4 tyrosine residues which may be responsible for the antigenic determinants [5].

To isolate peptides with immunoreactivity from ovalbumin, digestion of ovalbumin with trypsin must be performed in the presence of both 0.17% SDS and 23% glycerol. The former makes the conformation of ovalbumin loose and the latter makes it stable and protects trypsin. These concentrations of SDS and glycerol have been finally obtained after many trials of different combination of them. A small deviation from these concentrations makes it impossible for us to obtain peptides. Peptides A and B obtained retained still the antigenic determinants of ovalbumin owing

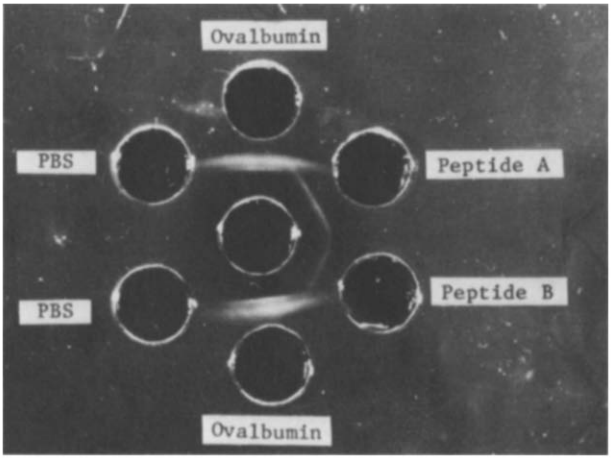

Fig.3. Immunodiffusion patterns of native ovalbumin and peptides A and B. In the central well: anti-ovalbumin serum.

not to a complete breaking of the conformation of the protein. This consideration may be proved by measuring the difference spectrum of ovalbumin in the presence and the absence of two reagents. The difference of ovalbumin with and without 0.17% SDS had sharp negative bands at around 287 and 294 nm due to the denaturation of the protein. Further addition there of 23% glycerol considerably reduced the spectral change, although glycerol itself did not affect the spectral of the protein. The success of the isolation of the peptides with immunoreactivity from ovalbumin may lead to not only the identification of the antigenic determining site but also the clarification of the mechanism of allergenic reaction induced by ovalbumin.

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